DECLARATION

I, Natsuo TANAKA, of HIRAKI & ASSOCIATES, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and Japanese languages and am competent to translate from Japanese into English.

2. That I have executed, with the best of my ability, a true and correct translation into English of Japanese Patent Application No. 2001-227510 filed on July 27, 2001, a copy of which I attach herewith.

This 6th day of April, 2005

N. Tanaka

Natsuo TANAKA

(Translation)

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[Title of the Invention] von Willebrand Factor (vWF)-Cleaving Protease

[Claim]

[Claim 1] A protease that is capable of cleaving a bond between residues Tyr-842 and Met-843 of von Willebrand factor (hereinafter referred to as "vWF") and comprises a polypeptide chain having the amino acid sequence Leu-Leu-Val-Ala-Val as a partial sequence or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

[Claim 2] The protease according to claim 1, which has molecular weight of 105 to 160 kDa in SDS-PAGE under reducing conditions.

[Claim 3] The protease according to claim 1 or 2, which comprises a polypeptide chain having the amino acid sequence Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val as the N-terminal partial sequence of a mature protein or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

[Claim 4] The protease according to any one of claims 1 to 3, which comprises a polypeptide chain having an amino acid sequence with deletion, substitution, or addition of one or several amino acids in the amino acid sequence as shown in SEQ ID NO: 3 or 7 or a partial sequence of any of the aforementioned amino acid sequences as the N-terminal partial sequence of a mature protein or the aforementioned amino acid sequence.

[Claim 5] The protease according to any one of claims 1 to 4, which comprises a polypeptide chain having an amino acid sequence with deletion, substitution, or addition of one or several amino acids in the amino acid sequence as shown in any of SEQ ID NOs: 16 to 21.

[Claim 6] A gene fragment encoding a protease that is capable of cleaving a bond between residues Tyr-842 and Met-843 of von Willebrand factor

(hereinafter referred to as "vWF") and comprises a polypeptide chain having the amino acid sequence Leu-Leu-Val-Ala-Val as a partial sequence or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

[Claim 7] A gene fragment encoding the protease according to any one of claims 2 to 5.

[Claim 8] DNA encoding the protease according to any one of claims 1 to 5, which comprises a nucleotide sequence encoding a polypeptide capable of cleaving a bond between residues Tyr 842 and Met 843 of vWF comprising CTG CTG GTG GCC GTG or with deletion, substitution, or addition of one or several nucleotides therein.

[Claim 9] The DNA encoding a protease according to claim 8, which comprises a nucleotide sequence comprising GCT GCA GGC GGC ATC CTA CAC CTG GAG CTG CTG GTG GCC GTG or with deletion, substitution, or addition of one or several nucleotides therein.

[Claim 10] The DNA encoding a protease according to claim 8 or 9, which comprises a nucleotide sequence with deletion, substitution or addition of one or several nucleotides in the nucleotide sequence as shown in SEQ ID NO: 6 or a partial sequence of any of the nucleotide sequences, or the nucleotide sequence.

[Claim 11] The DNA encoding a protease according to any one of claims 8 to 10, which comprises a nucleotide sequence with deletion, substitution or addition of one or several nucleotides in the nucleotide sequence as shown in SEQ ID NO: 15 or a partial sequence of any of the nucleotide sequences, or the nucleotide sequence.

[Claim 12] A pharmaceutical composition comprising the protease according to any one of claims 1 to 5.

[Claim 13] An antibody against the protease according to any one of claims 1 to 5.

[Claim 14] A pharmaceutical composition or diagnostic agent comprising an antibody against the protease according to any one of claims 1 to 5.

[Claim 15] A pharmaceutical composition or diagnostic agent comprising the DNA according to any one of claims 8 to 10 or antisense DNA thereof.

[Claim 16] A process for assaying vWF-cleaving activity, wherein a protease-substrate reaction is carried out using vWF and vWF-cleaving protease on a membrane filter, and a substrate sample is then recovered from the filter, followed by SDS-PAGE analysis without Western blotting.

[Claim 17] A process for preparing the protease according to any one of claims 1 to 5, wherein human plasma fraction I paste is used as a starting material.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a plasma protein related to the field of medical drugs. More particularly, the present invention relates to a protease that specifically cleaves von Willebrand factor (it may be hereafter referred to as "vWF"), which is associated with blood coagulation. The vWF-cleaving protease of the present invention enables replacement therapy for patients with diseases resulting from defects or decreases in this protease, such as thrombotic thrombocytopenic purpura (it may be hereafter referred to as "TTP"). In addition, the use thereof as a novel antiplatelet thrombotic agent is expected.

[0002]

[Prior Arts and Problems to be Solved by the Invention]

vWF is produced in vascular endothelial cells or megakaryocytes, and is a blood coagulation factor in which a single subunit comprising 2,050 amino acid residues (monomers of about 250 kDa) are bound by an S-S bond to form a multimer structure

(with a molecular weight of 500 to 20,000 kDa). The level thereof in the blood is about 10 μg/ml, and a high-molecular-weight factor generally has higher specific activity.

vWF has two major functions as a hemostatic factor. One of the functions is as a carrier protein wherein vWF binds to the blood coagulation factor VIII to stabilize it. Another function is to form platelet plug by adhering and agglomerating platelets on the vascular endothelial subcellular tissue of a damaged vascular wall.

[0003]

Thrombotic thrombocytopenic purpura is a disease that causes platelet plug formation in somatic arterioles and blood capillaries throughout the whole body. In spite of recent advances in medical technology, the morbidity associated with this disease approximately tripled from 1971 to 1991. Pathologically, TTP is considered to result from vascular endothelial cytotoxicity or vascular platelet aggregation. Immunohistologically, a large amount of vWFs are recognized in the resulting platelet plugs, and vWF is considered to play a major role in causing them. A normal or high-molecular-weight vWF multimer structure is dominant in a TTP patient, and an unusually large vWF multimer (ULvWFM) or large vWF multimer (LvWFM) is deduced to play a major role in accelerating platelet aggregation or microthrombus formation under high shearing stress. In contrast, vWF was known to degrade at a position between residues Tyr 842 and Met 843 by the action of vWF-cleaving protease in the circulating blood of a healthy person under high shearing stress. Accordingly, TTP is considered to occur in the following manner. The protease activity in the plasma is lowered for some reason, and ULvWFM to LvWFM are increased to accelerate platelet aggregation. This forms platelet plugs in blood vessels.

[0004]

Recently, Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918) and Tsai et al. (Blood, vol. 87, 4235-4244: 1996) developed a method for assaying vWF-specific cleaving protease. In their report, this protease activity was actually lowered in TTP. The aforementioned authors reported that this enzyme was metalloprotease in the plasma and partially purified. However, they have

not yet succeeded in the amino acid sequencing which would specify the protease. There have been no further developments since then.

[0005]

[Problems for Solving the Problem]

Up to the present, plasmapheresis therapy has been performed for treating patients who congenitally lack vWF-specific cleaving protease and patients who had acquired positive antibodies against this protease. Establishment of replacement therapy using purified products or a pure substance such as a recombinant gene product of the aforementioned protease is desired. Familial TTP patients congenitally lack vWF-specific cleaving protease, and non-familial TTP is caused by posteriori production of autoantibodies against the aforementioned protease. Accordingly, replacement therapy for this protease is preferable for familial TTP patients (plasma administration is actually performed), and removal of autoantibodies by plasmapheresis and substitution of this protease are necessary for non-familial TTP. Further, the use of this protease as a novel antiplatelet thrombotic agent can also be expected.

As mentioned above, however, Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918) and Tsai et al. (Blood, vol. 87, 4235-4244: 1996) have suggested that the vWF-cleaving protease was metalloprotease in the plasma. It was reported to be partially purified, and concentrated 1,000- to 10,000-fold from the plasma in terms of its specific activity. Even under these conditions, there has been no advancement in the analysis of the properties of this protease, such as the amino acid sequence of its protein, over the period of roughly 5 years that has passed since then. No specific biological information has yet been obtained regarding this protease. As reported by Furlan et al., the protein of interest is supposed to be gigantic, and there may be various problems associated therewith. For example, diversified forms of this protease, such as various interacting molecules or cofactors, are expected. Based on the complexity of purification processes, deteriorated capacity of separation by nonspecific interaction during the purification step, and other factors, it is deduced to be very difficult to isolate and identify the protease

from a plasma fraction by the purification process according to Furlan et al.

[0006]

[Means for Solving the Problem and Embodiments of the Invention]

Under the above circumstances, the present inventors have conducted concentrated studies in order to isolate and identify the vWF-cleaving protease. As a result, they have succeeded in isolating and purifying the vWF-cleaving protease of interest, which had not yet been reported. Thus, they have succeeded in identifying an amino acid sequence of the mature protein and a gene encoding this amino acid sequence.

The vWF-cleaving protease of the present invention can cleave a bond between residues Tyr 842 and Met 843 of vWF. Preferably, this protease has a molecular weight of 105 to 160 kDa in SDS-PAGE under reducing conditions. It is comprised of a polypeptide chain having Leu-Leu-Val-Ala-Val as a partial sequence. More preferably, it is comprised of a polypeptide chain having the partial N-terminal amino acid sequence of a mature protein, i.e., Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val. It is a novel substance characterized by the following properties.

[0007]

1) vWF-cleaving activity

According to the N-terminal sequence analysis of the cleavage fragment, the protease of the present invention cleaves a peptide bond between residues Tyr 842 and Met 843.

2) Fractionation by gel filtration

When fractionation is performed by gel filtration chromatography, most activities are collected in a fraction with a molecular weight of 150 to 300 kDa. According to one embodiment of the present invention, an actually obtained active substance is found to have a molecular weight of about 105 to 160 kDa in electrophoresis. Accordingly, the protease of the present invention is a substance that is likely to form a dimer or the like or to bind to another molecule.

3) Ammonium sulfate precipitation

A large portion of this protease is recovered as a precipitation fraction from a roughly purified fraction with the use of 33% saturated ammonium sulfate.

4) SDS-PAGE

The protease of the present invention has a molecular size of about 105 to 160 kDa determined by a molecular weight marker in SDS-PAGE in both of reduced condition or non-reduced condition. Further, this protease could be partially recovered in an active state after SDS-PAGE under non-reducing conditions.

[0008]

5) Analysis of amino acid sequence

The amino acid sequence of the isolated polypeptide fragment was analyzed. This presented an example of a polypeptide chain having a sequence Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val as a N-terminal amino acid sequence of a mature protein. Further, with current bioinformatics (BIOINFORMATICS: A Practical Guide to the Analysis of Genes and Proteins, edited by Andreas D. Baxevanis and B. F. Francis Ouellette), a nucleic acid sequence encoding the amino acid sequence was highly accurately identified by searching a database based on the aforementioned partial sequence. More specifically, the genome database was searched by the tblastn program. This identified a chromosome clone (AL158826) that is deduced to encode the protease of the present invention. Further, clones (AI346761 and AJ011374) that are deduced to be a part of the protease of interest and a part of the polypeptide to be encoded by the aforementioned genome were identified through collation with the Expressed Sequence Tag (EST) database. Based thereon, the amino acid sequence as shown in SEQ ID NO: 3 or 7 was identified as an active vWF-cleaving protease site.

[0009]

GCT GCA GGC GGC ATC CTA CAC CTG GAG CTG CTG GTG GCC GTG, a sequence deduced from the genome, and more preferably CTG CTG GTG GCC GTG, a portion thereof, the transcriptome of which was confirmed by EST, was obtained. The obtained nucleotide sequence was analyzed, and motif analysis was carried out based on

the deduced sequence. As a result, it was found to have a metalloprotease domain as a candidate for the protease of the present invention. Based on the above findings, it became possible to disclose a sequence of a polypeptide chain as a more specific example of the protease. Also, activities of proteases are generally known to vary depending on, for example, substitution, deletion, insertion, or introduction of point mutation into a portion of the amino acid sequence (Blood coagulation factor VII mutants, Soejima et al., JP Patent Publication (Kokai) No. 2001-61479 A). Similarly, the protease of the present invention can be modified by, for example, deletion, substitution, or addition of one or several amino acids, to prepare optimized proteases.

[0010]

The protease proteins were further mass-produced, and 29 amino acid sequences from the N-terminus were determined. These amino acid sequences are shown in SEQ ID NO: 8. This result is substantially the same as the sequence as shown in SEQ ID NO: 3 or 7 deduced by bioinformatics. Only one difference is that the amino acid 27th in SEQ ID NO: 3 or 7 was Glu while it was Arg according to the present analysis of the N-terminal sequence. This was considered to be a gene polymorphism. Thus, this protease was confirmed to be comprised of a polypeptide chain having the amino acid sequence as shown in SEQ ID NO: 3 or 7 at its N-terminus as a mature unit. A gene fragment encoding this protease was then cloned in the following manner.

[0011]

Based on the nucleic acid sequence as shown in SEQ ID NO: 7, a sense primer (SEQ ID NO: 9) and an antisense primer (SEQ ID NO: 10) were prepared based on the nucleic acid sequence underlined in Fig. 9, and a gene sandwiched between these primers was amplified. This fragment was cloned, and the nucleotide sequence was then confirmed. This fragment was used as a probe for Northern blotting to analyze the site at which the protease gene was expressed. As a result, this protease gene was found to be expressed

mainly in the liver. Accordingly, the human liver cDNA library was purchased, and a gene encoding this protease was identified using a rapid amplification of cDNA ends (RACE) technique. Based on these results, in the case of the largest sequence of approximately 5 kb of mRNA (cDNA) reaching the poly(A) addition site as shown in SEQ ID NO: 15 was identified.

[0012]

Based on the amino acid sequence deduced from this gene sequence, this protease was deduced to have a preprosequence, and to belong to the disintegrin and metalloprotease (ADAM) family having a disintegrin-like domain, a metalloprotease domain, and the like, and particularly to the ADAM-TS family having a thrombospondin Type-1 (TSP-1) domain. Finally, including those having insertion or deletion in a part of the nucleic acid sequence, isoforms as shown in SEQ ID NOs: 16 to 21 having sequences as shown in SEQ ID NOs: 3 and 7 at the N-terminuses after the mature preprosequence has been cleaved were identified.

[0013]

The vWF-cleaving protease of the present invention can be generally prepared by the following process.

According to the present invention, a process for assaying the protease activity is characterized by the possibility of evaluating activity within a short period of time. According to the report by Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918 A), activity is assayed by analyzing vWF-cleaving patterns by Western blotting using the anti-vWF antibody, and thus, it takes time to transfer the protease to a filter. More specifically, this process requires approximately at least 45 hours in total, i.e., 24 hours for the enzymatic reaction with a substrate vWF, 17 hours for electrophoresis, and 3 hours to transfer the protease to a filter, followed by detection using the anti-vWF antibody. In contrast, the present inventors completed activity assay in 18 hours in total, i.e., 16 hours for the enzymatic

reaction with a substrate vWF, and 2 hours for electrophoresis and detection. This indicates that the time required for the assay can be reduced to one third or less of that required for the conventional assay. This can also shorten the time required for the purification process, and in turn can lower the degree of the protease to be inactivated. Accordingly, purification efficiency is improved compared with that attained by the method of Furlan et al., and as a result, the degree of purification is also enhanced.

[0014]

Further, the starting material was examined using the aforementioned assay system. As a result, it was found that the protease activity was more concentrated in FI paste than in the cryoprecipitate that had been reported by Furlan et al. in the past. FI paste was used as a starting material, and the aforementioned rapid activity assay systems were combined. This enabled isolation and identification of the protease of interest. In a specific embodiment, a purification process combining gel filtration chromatography with ion exchange chromatography is employed, and the aforementioned activity assay system is also combined.

More specifically, FI paste is solubilized with a buffer, and the resultant is fractionated by gel filtration chromatography. The protease activity is fractionated at the elution region with a molecular weight of 150 to 300 kDa deduced from the size marker of gel filtration. Thereafter, the resultant is precipitated and concentrated using 33% saturated ammonium sulfate. This procedure is repeated three times in total. The active fraction obtained in the third gel filtration is pooled, and the resultant is subjected to dialysis at 4°C overnight with a buffer comprising 50 mM NaCl added to 50 mM Tris-HCl (pH 7.1). Thereafter, the dialysis product is subjected to anion exchange chromatography (DEAE) and eluted stepwise with 0.25 M NaCl. The present inventors have conducted concentrated studies in order to find a process for isolating and identifying the protease of the present invention.

As a result, they found that, surprisingly, the protease was recoverable as an active band after non-reducing SDS-PAGE. In order to achieve further mass production, the purified and concentrated fraction was applied to the Biophoresis utilizing the principle of SDS-PAGE. Thus, a fraction having vWF-cleaving activity was isolated from the electrophoresed fraction. According to the approximate calculation of the specific activity up to this phase, purification of about 30,000- to 100,000-fold was achieved. This procedure was efficiently and rapidly repeated several times, and thus, about 0.5 pmole of sample that is the current limit of the analysis of amino acid sequence was obtained. Thus, analysis of amino acid sequence became feasible. More specifically, a final step of separation and purification (Biophoresis) based on the principle of SDS-PAGE is important, and it is based on the findings as a result of concentrated studies, which had led to the completion of the present invention.

[0015]

According to the report by Furlan et al., specific activity was improved by as much as about 10,000 times, although the protease was not substantially isolated or identified. This could be because of deactivation during purification or the difficulty of isolating and identifying molecules, which were gigantic proteins capable of interacting with various other proteins such as the protease of the present invention by a separation method utilizing various types of liquid chromatography. Further, the protease content in the plasma was deduced to be very small, and thus, it was necessary to await the establishment of the process according to the present invention.

[0016]

Based on the findings of the present invention, peptides or proteins prepared from the obtained sequences are determined to be antigens. With the use thereof, a monoclonal antibody, a polyclonal antibody, or a humanized

antibody thereof can be prepared by general immunization techniques (Current Protocols in Molecular Biology). These antibodies can be applied to diagnosis and therapy of diseases such as TTP.

[0017]

Based on the obtained genome or EST sequence, cDNA or a genomic gene encoding the protease of the present invention can be cloned by a common technique (Molecular Cloning, 2nd edition). These genes are incorporated into a suitable expression vector, the resultant is transformed into a suitable host cell, and the gene recombinant product of the protease can be thus prepared. Based on the gene sequence of the above protease, a probe, primer, or antisense is designed by a common technique. This enables the gene diagnosis or gene therapy. The peptide or protein of the present invention is used as a leading substance for amino acid modification. This enables the preparation of a molecule having activity that is different from that of the protease of the present invention.

[0018]

[Advantage of the Invention]

The findings of the present invention have led to the possibility of replacement therapy for patients having diseases resulting from deficiency of a protease, such as thrombotic thrombocytopenic purpura. This also realizes the establishment of methods for gene cloning and efficient purification from serum or plasma. In particular, the information provided by the present invention enables gene recombination based on the obtained nucleotide sequence and stable production and provision of the protease according to the present invention, which have been heretofore difficult to achieve. Also, these can be applied to replacement therapy for TTP patients, inhibition of platelet plug formation. Diagnosis and therapy utilizing the gene encoding the protease of the present invention or an antibody thereagainst can be realized.

[0019]

The present invention is hereafter described in detail with reference to

the following examples, although it is not limited to these examples.

[Examples]

Example 1

(Preparation of vWF)

A plasma cryoprecipitation (2 g) was dissolved in 20 ml of buffer (0.01% Tween-80/50 mM Tris-HCl/100 mM NaCl, pH 7.4), and the resultant was subjected to gel filtration using a Sephacryl S-500 HR Column (2.6 x 90 cm, Amersham Pharmacia) to prepare vWF. Fractions were recovered at a flow rate of 2 ml/min in amounts of 6 ml each. vWF was analyzed by Western blotting using a peroxidase-labeled rabbit anti-human vWF antibody (DAKO), and high-molecular-weight vWF fractions were pooled. The pooled fractions were subjected to multimer analysis using agarose electrophoresis as described below.

As shown in Fig. 1, vWF originally has a multimer structure in which vWF monomer molecules are polymerized with each other at their N-terminuses or at their C-terminuses, and vWF is subjected to partial hydrolysis by the vWF-specific cleaving protease. As a result of the analysis, as shown in Fig. 2, the purified vWF exhibited a multimer pattern based on agarose electrophoresis approximately equivalent to that in the plasma of a healthy person (the ladder in the drawing shows the electrophoresis pattern of vWF having a multimer structure, and the upper portion indicates vWF with advanced polymerization). This can prepare vWF comprising substantially no impurities that degrade it, and this fraction was used as a substrate when assaying the vWF-cleaving activity as described below.

[0020]

Example 2

(vWF-cleaving reaction)

vWF-cleaving activity was assayed as follows. A sample comprising 10 mM barium chloride (final concentration) was pre-incubated at 37°C for 5 minutes to activate protease. A buffer (15 to 20 ml, 1.5 M urea/5 mM Tris-HCl, pH 8.0) was placed in a 50 ml Falcon Tube. Subsequently, a membrane filter (0.025 μm, Millipore) was floated

therein, and 100 μl of activated sample prepared by mixing with 50 μl of vWF substrate solution was added. The resultant was allowed to stand in an incubator (37°C) overnight and recovered from the filter on the next day. The recovered sample was evaluated based on the vWF cleavage pattern as described below in the "SDS-PAGE" section.

[0021]

SDS-PAGE

SDS-5% polyacrylamide gel was autologously prepared and used. An SDS electrophoresis buffer (2 μl, in the presence or absence of a reducing agent, i.e., 2-mercaptoethanol) was added to 10 μl of the sample described in the "vWF-cleaving activity assay" section, and the resultant was boiled for 3 minutes to prepare an electrophoresis sample. The gel was subjected to electrophoresis at 30 mA for 1 hour and then stained with the Gel Code Blue Stain Reagent (PIERCE) utilizing CBB staining. As shown in Fig. 1, activity is evaluated based on the development of a cleavage fragment and the presence or absence of fragments remaining uncleaved under reducing or non-reducing conditions. This is more specifically described in Example 3 and Fig. 3 below.

[0022]

Multimer analysis utilizing agarose electrophoresis

Preparation of gel, electrophoresis

Low gelling temperature agarose (Type VII, Sigma) was added to 375 mM Tris-HCl (pH 6.8) until a concentration of 1.4% was reached, followed by heating in a microwave oven to completely dissolve the gel. Thereafter, 0.1% SDS was added, and the resultant was maintained at 56°C. The resultant was made to flow into a gel mold and solidified by cooling at 4°C overnight (running gel). The next day, high gelling temperate agarose (SeaKem) was mixed with 375 mM Tris-HCl (pH 6.8) until a concentration of 0.8% was reached, and dissolved by boiling in a microwave oven. Thereafter, the resultant was maintained at 56°C (stacking gel). The gel prepared on the previous day was cleaved, leaving a 10-cm fraction from the end uncleaved. The

aforementioned gel was made to flow into the cleaved portion, and the gel was made to keep flowing at 4°C for at least 3 hours, followed by solidification. Pyronin Y was added to the sample described in the "vWF cleaving activity assay" section above, and the gel was prepared under non-reducing conditions without boiling. The gel was subjected to electrophoresis at 10 mA for at least 24 hours using an SDS-PAGE buffer.

[0023]

Western blotting

After the electrophoresis, the gel was immersed in a transcription buffer (0.005% SDS, 50 mM phosphate buffer, pH 7.4) for 10 minutes, and the resultant was transferred to a nitrocellulose membrane using a transcription apparatus at 4°C at 0.5 A overnight. Blocking was performed using a blotting solution (5% skim milk, PBS) for 30 minutes, and the gel was then allowed to react for at least 6 hours with the peroxidase-labeled rabbit anti-human vWF antibody (DAKO), which was diluted 1,000-fold with the blotting solution. Thereafter, the gel was washed three times with the blotting solution and once with PBS, and color was developed using Konica Immunostain HRP-1000 (Konica), which was a substrate reaction solution for peroxidase. The purified vWF analyzed in this assay was found to have been undegraded, but was sufficiently usable as a substrate in the present invention (Fig. 2).

[0024]

Example 3

(Preparation of vWF-cleaving protease)

Plasma was subjected to ethanol fractionation developed by Cohn. A protease having high vWF-cleaving activity (one with high specific activity) when protein levels in four fractions (i.e., starting plasma, cryoprecipitate, fraction I (FI) supernatant, and a paste) are made equivalent to each other was selected. As shown in Fig. 3, the protease activity was highest in the FI paste. The N-terminal sequence of this cleavage fragment was analyzed, and as a result, activity derived from the cryoprecipitate and the FI paste were found to cleave the peptide bond between residues Tyr 842 and Met 843. Thus, the FI paste was determined to be a main starting material for purification thereafter.

[0025]

Solubilization of FI paste

The FI paste was fractionated in fractions of 12 g each and then cryopreserved. The paste was allowed to melt at 4°C the day before its use. The next day, 120 ml of solubilizing buffer (0.05% azide, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl) was added at 10 mg/ml, and the mixture was stirred at 37°C for 2 hours. The product was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was then recovered, followed by filtration with a prefilter, a 5.0 μm filter, and a 0.8 μm filter in that order. The resultant was determined to be a solubilized sample. Fig. 4 shows the result of SDS-PAGE of the solubilized sample.

[0026]

Gel filtration chromatography of vWF-cleaving protease

The solubilized F1 paste was applied to a Sephacryl S-300 HR Column (5 x 90 cm, Amersham Pharmacia) to conduct the first gel filtration. A buffer comprising 0.05% azide, 50 mM Tris-HCl (pH 7.4), and 100 mM NaCl (hereinafter referred to as an "elution buffer"), which was the same as the solubilizing buffer, was used. The flow rate was 5 ml/min, fractionation was initiated at 600 ml after the sample application, and fractions were recovered in amounts of 10 ml each. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE. Fractions that exhibited protease activity were pooled, and a small amount of saturated ammonium sulfate was gradually added dropwide thereto until a final concentration of 33% saturation was reached. The mixture was further allowed to stand at 4°C overnight. The next day, the product was centrifuged at 10,000 rpm for 10 minutes, and an active fraction of interest was recovered as a precipitate. The procedures comprising solubilization, gel filtration, and ammonium sulfate precipitation were performed for 5 batches and the resultant was cryopreserved at -20°C.

[0027]

The ammonium sulfate precipitates (2 to 3 batches) obtained by the first gel filtration were dissolved in 50 ml of elution buffer, and passed through the Sephacryl

S-300 HR Column (5 x 90 cm) in the same manner as in the first gel filtration to perform the second gel filtration. The elution buffer, conditions, operations, and the like were the same as those in the first gel filtration. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE. Fractions with activity were pooled, and ammonium sulfate precipitation was similarly performed. These procedures were repeated two times.

[0028]

The ammonium sulfate precipitates (2 batches) obtained by the second gel filtration were dissolved in 50 ml of elution buffer, and applied to the Sephacryl S-300 HR Column (5 x 90 cm) in the same manner as in the first and the second gel filtration to perform the third gel filtration. The elution buffer, conditions, operations, and the like were the same as those in the first and the second gel filtration. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE, followed by pooling. Fig. 5 shows SDS-PAGE for analyzing these fractions and that for analyzing vWF-cleaving activity. Based on the patterns of gel filtration and the data showing activity, the protease of the present invention was found to be eluted in the region between fraction 37 and fraction 47. Based on a separately conducted elution experiment for high-molecular-weight gel filtration marker (Amersham Pharmacia), this site of elution was deduced to have a molecular weight equivalent to 150 to 300 kDa. In this phase, considerable amounts of impurities were still present.

[0029]

DEAE anion exchange chromatography

The pooled fraction obtained by three gel filtration operations was subjected to dialysis overnight with a buffer comprising 50 mM Tris-HCl and 50 mM NaCl (pH 7.1). After the dialysis, anion exchange chromatography was performed using a 5 ml HiTrap DEAE-Sepharose Fast Flow Column (Pharmacia) to conduct further purification and concentration. Equilibrating and washing were performed using a buffer comprising 50 mM Tris-HCl (pH 7.1), and elution was performed using 0.25 M NaCl. The flow rate

was 5 ml/min, and 5 fractions of 5 ml each were recovered and pooled. Fig. 6 shows the results of SDS-PAGE for analyzing elution fractions and those for analyzing vWF-cleaving activity. Based on SDS-PAGE for activity assay, the protease of the present invention having vWF-cleaving activity was considerably effectively concentrated in the elution fraction.

[0030]

Fractionation utilizing SDS-PAGE

The sample (5 ml) purified and concentrated by DEAE anion exchange chromatography was further concentrated to 0.5 ml using Centricon (molecular weight cut off: 10,000 Da, Amicon). The protease of the present invention was isolated by Biophoresis III (Atto Corporation) utilizing SDS-PAGE. In accordance with the Laemmli method (Nature, vol. 227, 680-685, 1970), a buffer for electrophoresis tanks was prepared, and developed with 8% polyacrylamide gel to recover the electrophoresis fraction. Fig. 7 shows the result of SDS-PAGE for analyzing the recovered fractions. The buffer used for recovery was comprised of 50 mM Tris-HCl and 10% glycerol (pH 8.8). As is apparent from Fig. 7, this process according to the present invention has a high ability to produce separation. Fig. 8 shows the results of analyzing activity of a fraction further purified by electrophoresis and the results of SDS-PAGE for analyzing active fractions. The protease of the present invention can be recovered as an active molecule even after SDS-PAGE. When the activity of this protease in the plasma is determined to be 1 in terms of specific activity, a degree of purification of 30,000- to 100,000-fold was deduced to be achieved based on the average protein content in the plasma (60 mg/ml).

[0031]

Example 4

(Partial amino acid sequencing)

The partial amino acid sequence of the isolated protease was determined. This protease, which was isolated using Biophoresis, was transferred to a PVDF membrane after SDS-PAGE by a conventional technique, air-dried, and then subjected to analysis

using the automated protein sequencer (model 492; PE Applied Biosystems). As a result, the vWF-cleaving protease of the present invention isolated under the above conditions was found to comprise a polypeptide chain having a molecular weight of 105 to 160 kDa in SDS-PAGE under reducing conditions. This protease was also found to have, as a partial sequence, Leu-Leu-Val-Ala-Val, and preferably Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val.

[0032]

Deduction of isolated protease utilizing bioinformatics

At present, bioinformatics enables the deduction of full nucleotide sequences encoding a polypeptide without substantial gene cloning through collation with information in the database accumulated in the past (BIOINFORMATICS: A Practical Guide to the Analysis of Genes and Proteins, edited by Andreas D. Baxevanis and B. F. Francis Ouellette). Based on the partial amino acid sequencing by the aforementioned process (Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val), the database was searched by the tblastn program. As a result, a chromosome clone (AL158826) that was deduced to encode the protease of the present invention was identified by genomic database search. Further, a part of the protease of interest as the expressed sequence tag (EST) and a clone that was deduced to be a part of the polypeptide encoded by the aforementioned genome (AI346761 and AJ011374) were identified. The amino acid sequence as shown in SEQ ID NO: 3 or 7 was deduced based thereon to be an active vWF-cleaving protease site.

[0033]

Example 5

(Gene identification)

Synthesis of all the following synthetic primers was performed by Greiner Japan Co.Ltd. by request. Further, reagents used for gene recombination were those manufactured by TAKARA, TOYOBO, and New England Biolabs unless otherwise specified.

[0034]

Preparation of a gene fragment as a Northern blotting probe

A sense primer (SEQ ID NO: 9) and an antisense primer (SEQ ID NO: 10) were prepared. PCR was carried out using Universal QUICK-Clone™ cDNA (Clontech), which was a mixture of cDNA derived from normal human tissue, as a template and TaKaRa LA Taq with GC rich buffer. A gene sandwiched between these primers was amplified, and the amplified fragment was cloned using a TOPO TA cloning™ kit (Invitrogen). DNAs having the nucleotide sequence as shown in SEQ ID NO: 6 were isolated from several clones.

A vector portion was removed from this cloned DNA by EcoRI digestion, separated and purified by agarose electrophoresis, and the resultant was determined to be a template for preparing probes for Northern blotting.

[0035]

Northern blotting

The gene fragment prepared above was employed as a template to prepare a radioactive probe using [α-^{32}P]dCTP (Amersham Pharmacia) and a BcaBEST™ labeling kit (TAKARA). Hybridization was carried out using the Human 12-lane Multiple Tissue Northern Blots™ (Clontech) filter in accordance with the method described in Molecular Cloning 2nd Edition, pp. 9.52-9.55. Detection was carried out by autoradiography. As shown in Fig. 10, mRNA encoding the protease was expressed mainly in the liver. The size of this mRNA was found to be more than 4.4 kb.

[0036]

Isolation and identification of gene encoding the protease

As a result of Northern blotting, mRNA was found to be expressed mainly in the liver. Thus, the protease gene of the present invention was isolated and identified in accordance with the RACE technique using normal human liver-derived poly A$^+$ RNA and Marathon-Ready™ cDNA (Clontech).

More specifically, the first PCR was carried out as 5' RACE using normal human liver-derived Marathon-Ready™ cDNA in accordance with the product's manual and using the AP-1 primer attached to the kit and antisense primers (SEQ ID NOs: 11 to 13)

arbitrarily selected from the group of Gene Specific Primers (GSP) excluding the primer 1 located in the uppermost stream as shown in Fig. 11. Nested PCR (the second PCR) was then carried out using the AP-2 primer located in the inside thereof and the antisense primer located in the inside of the primer used for the first PCR as shown in Fig. 11. Thereafter, TA cloning was carried out. Genes were prepared from the developed colonies in accordance with a conventional technique (Molecular Cloning 2nd Edition, pp. 1.25-1.28), and nucleic acid sequences were decoded using an automatic DNA sequencer. The primer used for sequencing was the primer used for PCR or a primer located in the inside thereof. Further, the primer was designed based on the sequence determined after serial decoding.

[0037]

3' RACE was started from normal human liver-derived poly A^+ RNA using the 3'-Full RACE Core Set (TAKARA), and reverse transcription was carried out in accordance with the attached manual using the attached oligo dT primer. The band amplified by PCR using the sense primer (SEQ ID NO:14) located at "primer 2" in Fig. 11 and the attached oligo dT primer was separated by agarose electrophoresis and extracted, followed by TA cloning. Genes were prepared from the developed colonies, and nucleic acid sequences were decoded using an automatic DNA sequencer. A primer used for sequencing was designed based on the sequence determined after serial decoding.

[Brief description of the drawings]

[Figure 1] Fig.1 is a diagram showing the vWF multimer structure and the point cleaved by the vWF-cleaving protease.

[Figure 2] Fig.2 is a diagram showing the result of vWF multimer analysis (agarose electrophoresis).

[Figure 3] Fig.3 is a diagram showing the result of SDS-PAGE (5% gel) for analyzing the vWF-cleaving activity of each plasma fraction under reducing conditions.

[Figure 4] Fig.4 is a diagram showing the result of SDS-PAGE (5% gel) for

analyzing the solubilized sample of fraction 1 (F1) paste under non-reducing conditions.

[Figure 5] Fig.5 is a diagram showing the result of analyzing vWF-cleaving protease fractions after being subjected to gel filtration chromatography three times using the solubilized sample of F1 paste as a starting material.

[Figure 6] Fig.6 is a diagram showing the results of analyzing vWF-cleaving protease fractions in which the fraction collected by gel filtration chromatography is purified by DEAE anion exchange chromatography.

[Figure 7] Fig.7 is a diagram showing an electrophoresed fragment obtained when the vWF-cleaving protease fraction purified and concentrated by DEAE anion exchange chromatography is further purified by Biophoresis-based SDS-PAGE (non-reducing conditions).

[Figure 8] Fig.8 is a diagram showing the result of electrophoresis on a fraction obtained by further purifying a vWF-cleaving protease fraction by Biophoresis-based SDS-PAGE for analyzing vWF-cleaving protease activity and SDS-PAGE on active fractions under reducing conditions.

[Figure 9] Fig. 9 relates to the identification of the vWF-cleaving protease gene, which is a diagram showing primers used for amplifying the gene fragment for a Northern blot probe.

[Figure 10] Fig.10 relates to the identification of the vWF-cleaving protease gene, which is a photograph showing Northern blot autoradiography.

[Figure 11] Fig.11 relates to the identification of the vWF-cleaving protease gene, and is a diagram showing the locations and the sequences of the primers used in the RACE experiments.

[Document Name] Abstract

[Summary]

[Object] This invention is intended to isolate and identify a vWF-specific cleaving protease.

[Means for Solving the Problem] A desired vWF specific cleaving protease can be isolated and purified using Fraction I (FI) paste derived from human plasma as a starting material by purifying and concentrating steps comprising gel filtration, anion change chromatography, and SDS-PAGE based on Biophoresis. The vWF specific cleaving protease cleaves 842Tyr-843Met bond of vWF and has preferably molecular weight of 105-160 kDa in SDS-PAGE and consists of a polypeptide chain comprising a partial sequence of Leu-Leu-Val-Ala-Val.

[Advantageous Effect] The isolation and identification of the vWF specific cleaving enzyme will result in the expectation of replacement therapy for TTP patients and the like which are caused by the deficiency of the vWF specific cleaving enzyme.

[Selected Figure] Non

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Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly

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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu

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Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly

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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala

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gcg ccc cgc gcc ggc ctc gcc tgg tcc ccc tgc agc cgc cgg cag 585

Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln

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Gln Pro Gly Leu Tyr Tyr Ser Ala Asn Glu Gln Cys Arg Val Ala
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Phe Thr Tyr Phe Gln Pro Lys Pro Arg Gln Ala Trp Val Trp Ala
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Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
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Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
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Leu Arg Phe Leu Cys Met Asp Ser Ala Leu Arg Val Pro Val Gln
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Glu Glu Leu Cys Gly Leu Ala Ser Lys Pro Gly Ser Arg Arg Glu
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890 895 900

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Glu Ala Cys Ser Leu Glu Pro Cys Pro Pro Arg Trp Lys Val Met
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Ser Leu Gly Pro Cys Ser Ala Ser Cys Gly Leu Gly Thr Ala Arg
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cgc tcg gtg gcc tgt gtg cag ctc gac caa ggc cag gac gtg gag          2925
Arg Ser Val Ala Cys Val Gln Leu Asp Gln Gly Gln Asp Val Glu
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Val Pro Cys Leu Ile Ala Asp Cys Thr Tyr Arg Trp His Val Gly
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cgg cgt gac acc tgc ctc gga ccc cag gcc cag gcg cct gtg cca          3105
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gct gat ttc tgc cag cac ttg ccc aag ccg gtg act gtg cgt ggc          3150
Ala Asp Phe Cys Gln His Leu Pro Lys Pro Val Thr Val Arg Gly
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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val
            20                  25                  30
ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc         135
Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
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Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
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Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser
            65                  70                  75
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Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
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Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
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Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala
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Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln
                185                 190                 195
ctg ctg agc ctg ctc agg acg ggc gcg ctg cgt gtg gga ccc gcc      630
Leu Leu Ser Leu Leu Arg Thr Gly Ala Leu Arg Val Gly Pro Ala
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Ala Ala Ser Thr Arg Val Arg Gly Ala Pro Ala Gly Cys Ala Ala
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Trp Pro Leu Leu Gln Arg Gln Arg Ala Val Pro Arg Gly Leu Arg
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Pro Gln Gly Cys Arg Leu His Leu Arg Gln Gly Ala Pro Gly Glu
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Trp Cys Ser Lys Gly Arg Cys Arg Ser Leu Val Glu Leu Thr Pro

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380 385 390

aag acc cag ctg gag ttc atg tcg caa cag tgc gcc agg acc gac 1215

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Gly Gln Pro Leu Arg Ser Ser Pro Gly Gly Ala Ser Phe Tyr His

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425 430 435
cac atg tgc cgg gcc att ggc gag agc ttc atc atg aag cgt gga 1350
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200 205 210

gcg gcc tca acc cgg gtc cgc ggg gca ccc gcc gga tgc gca gcc 675
Ala Ala Ser Thr Arg Val Arg Gly Ala Pro Ala Gly Cys Ala Ala
215 220 225

tgg cct cta cta cag cgc caa cga gca gtg ccg cgt ggc ctt cgg 720
Trp Pro Leu Leu Gln Arg Gln Arg Ala Val Pro Arg Gly Leu Arg
230 235 240

ccc caa ggc tgt cgc ctg cac ctt cgc cag gga gca cct ggt gag 765
Pro Gln Gly Cys Arg Leu His Leu Arg Gln Gly Ala Pro Gly Glu
245 250 255

tct gcc ggc ggt ggc ctg gga ttg gct gtg agg tcc ctc cgc atc 810
Ser Ala Gly Gly Gly Leu Gly Leu Ala Val Arg Ser Leu Arg Ile
260 265 270

```
acc cag ctc acg tcc ccc caa acg tgc atg gat atg tgc cag gcc        855
Thr Gln Leu Thr Ser Pro Gln Thr Cys Met Asp Met Cys Gln Ala
                275                 280                 285
ctc tcc tgc cac aca gac ccg ctg gac caa agc agc tgc agc cgc        900
Leu Ser Cys His Thr Asp Pro Leu Asp Gln Ser Ser Cys Ser Arg
                290                 295                 300
ctc ctc gtt cct ctc ctg gat ggg aca gaa tgt ggc gtg gag aag        945
Leu Leu Val Pro Leu Leu Asp Gly Thr Glu Cys Gly Val Glu Lys
                305                 310                 315
tgg tgc tcc aag ggt cgc tgc cgc tcc ctg gtg gag ctg acc ccc        990
Trp Cys Ser Lys Gly Arg Cys Arg Ser Leu Val Glu Leu Thr Pro
                320                 325                 330
ata gca gca gtg cat ggg cgc tgg tct agc tgg ggt ccc cga agt        1035
Ile Ala Ala Val His Gly Arg Trp Ser Ser Trp Gly Pro Arg Ser
                335                 340                 345
cct tgc tcc cgc tcc tgc gga gga ggt gtg gtc acc agg agg cgg        1080
Pro Cys Ser Arg Ser Cys Gly Gly Gly Val Val Thr Arg Arg Arg
                350                 355                 360
cag tgc aac aac ccc aga cct gcc ttt ggg ggg cgt gca tgt gtt        1125
Gln Cys Asn Asn Pro Arg Pro Ala Phe Gly Gly Arg Ala Cys Val
                365                 370                 375
ggt gct gac ctc cag gcc gag atg tgc aac act cag gcc tgc gag        1170
Gly Ala Asp Leu Gln Ala Glu Met Cys Asn Thr Gln Ala Cys Glu
                380                 385                 390
aag acc cag ctg gag ttc atg tcg caa cag tgc gcc agg acc gac        1215
Lys Thr Gln Leu Glu Phe Met Ser Gln Gln Cys Ala Arg Thr Asp
                395                 400                 405
ggc cag ccg ctg cgc tcc tcc cct ggc ggc gcc tcc ttc tac cac        1260
```

Gly Gln Pro Leu Arg Ser Ser Pro Gly Gly Ala Ser Phe Tyr His

410 415 420

tgg ggt gct gct gta cca cac agc caa ggg gat gct ctg tgc aga 1305

Trp Gly Ala Ala Val Pro His Ser Gln Gly Asp Ala Leu Cys Arg

425 430 435

cac atg tgc cgg gcc att ggc gag agc ttc atc atg aag cgt gga 1350

His Met Cys Arg Ala Ile Gly Glu Ser Phe Ile Met Lys Arg Gly

440 445 450

gac agc ttc ctc gat ggg acc cgg tgt atg cca agt ggc ccc cgg 1395

Asp Ser Phe Leu Asp Gly Thr Arg Cys Met Pro Ser Gly Pro Arg

455 460 465

gag gac ggg acc ctg agc ctg tgt gtg tcg ggc agc tgc agg aca 1440

Glu Asp Gly Thr Leu Ser Leu Cys Val Ser Gly Ser Cys Arg Thr

470 475 480

ttt ggc tgt gat ggt agg atg gac tcc cag cag gta tgg gac agg 1485

Phe Gly Cys Asp Gly Arg Met Asp Ser Gln Gln Val Trp Asp Arg

485 490 495

tgc cag gtg tgt ggt ggg gac aac agc acg tgc agc cca cgg aag 1530

Cys Gln Val Cys Gly Gly Asp Asn Ser Thr Cys Ser Pro Arg Lys

500 505 510

ggc tct ttc aca gct ggc aga gcg aga gaa tat gtc acg ttt ctg 1575

Gly Ser Phe Thr Ala Gly Arg Ala Arg Glu Tyr Val Thr Phe Leu

515 520 525

aca gtt acc ccc aac ctg acc agt gtc tac att gcc aac cac agg 1620

Thr Val Thr Pro Asn Leu Thr Ser Val Tyr Ile Ala Asn His Arg

530 535 540

cct ctc ttc aca cac ttg gcg gtg agg atc gga ggg cgc tat gtc 1665

Pro Leu Phe Thr His Leu Ala Val Arg Ile Gly Gly Arg Tyr Val

545 550 555

gtg gct ggg aag atg agc atc tcc cct aac acc acc tac ccc tcc 1710
Val Ala Gly Lys Met Ser Ile Ser Pro Asn Thr Thr Tyr Pro Ser
560 565 570

ctc ctg gag gat ggt cgt gtc gag tac aga gtg gcc ctc acc gag 1755
Leu Leu Glu Asp Gly Arg Val Glu Tyr Arg Val Ala Leu Thr Glu
575 580 585

gac cgg ctg ccc cgc ctg gag gag atc cgc atc tgg gga ccc ctc 1800
Asp Arg Leu Pro Arg Leu Glu Glu Ile Arg Ile Trp Gly Pro Leu
590 595 600

cag gaa gat gct gac atc cag gtt tac agg cgg tat ggc gag gag 1845
Gln Glu Asp Ala Asp Ile Gln Val Tyr Arg Arg Tyr Gly Glu Glu
605 610 615

tat ggc aac ctc acc cgc cca gac atc acc ttc acc tac ttc cag 1890
Tyr Gly Asn Leu Thr Arg Pro Asp Ile Thr Phe Thr Tyr Phe Gln
620 625 630

cct aag cca cgg cag gcc tgg gtg tgg gcc gct gtg cgt ggg ccc 1935
Pro Lys Pro Arg Gln Ala Trp Val Trp Ala Ala Val Arg Gly Pro
635 640 645

tgc tcg gtg agc tgt ggg gca ggg ctg cgc tgg gta aac tac agc 1980
Cys Ser Val Ser Cys Gly Ala Gly Leu Arg Trp Val Asn Tyr Ser
650 655 660

tgc ctg gac cag gcc agg aag gag ttg gtg gag act gtc cag tgc 2025
Cys Leu Asp Gln Ala Arg Lys Glu Leu Val Glu Thr Val Gln Cys
665 670 675

caa ggg agc cag cag cca cca gcg tgg cca gag gcc tgc gtg ctc 2070
Gln Gly Ser Gln Gln Pro Pro Ala Trp Pro Glu Ala Cys Val Leu
680 685 690

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Glu Pro Cys Pro Pro Tyr Trp Ala Val Gly Asp Phe Gly Pro Cys
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Ser Ala Ser Cys Gly Gly Gly Leu Arg Glu Arg Pro Val Arg Cys
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Val Glu Ala Gln Gly Ser Leu Leu Lys Thr Leu Pro Pro Ala Arg
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Cys Arg Ala Gly Ala Gln Gln Pro Ala Val Ala Leu Glu Thr Cys
                740                 745                 750
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Asn Pro Gln Pro Cys Pro Ala Arg Trp Glu Val Ser Glu Pro Ser
                755                 760                 765
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Ser Cys Thr Ser Ala Gly Gly Ala Gly Leu Ala Leu Glu Asn Glu
                770                 775                 780
acc tgt gtg cca ggg gca gat ggc ctg gag gct cca gtg act gag          2385
Thr Cys Val Pro Gly Ala Asp Gly Leu Glu Ala Pro Val Thr Glu
                785                 790                 795
ggg cct ggc tcc gta gat gag aag ctg cct gcc cct gag ccc tgt          2430
Gly Pro Gly Ser Val Asp Glu Lys Leu Pro Ala Pro Glu Pro Cys
                800                 805                 810
gtc ggg atg tca tgt cct cca ggc tgg ggc cat ctg gat gcc acc          2475
Val Gly Met Ser Cys Pro Pro Gly Trp Gly His Leu Asp Ala Thr
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tct gca ggg gag aag gct ccc tcc cca tgg ggc agc atc agg acg          2520
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845 850 855

tcc gtc tcc tgc ggg cga ggt ctg atg gag ctg cgt ttc ctg tgc 2610
Ser Val Ser Cys Gly Arg Gly Leu Met Glu Leu Arg Phe Leu Cys
860 865 870

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Met Asp Ser Ala Leu Arg Val Pro Val Gln Glu Glu Leu Cys Gly
875 880 885

ctg gca agc aag cct ggg agc cgg cgg gag gtc tgc cag gct gtc 2700
Leu Ala Ser Lys Pro Gly Ser Arg Arg Glu Val Cys Gln Ala Val
890 895 900

ccg tgc cct gct cgg tgg cag tac aag ctg gcg gcc tgc agc gtg 2745
Pro Cys Pro Ala Arg Trp Gln Tyr Lys Leu Ala Ala Cys Ser Val
905 910 915

agc tgt ggg aga ggg gtc gtg cgg agg atc ctg tat tgt gcc cgg 2790
Ser Cys Gly Arg Gly Val Val Arg Arg Ile Leu Tyr Cys Ala Arg
920 925 930

gcc cat ggg gag gac gat ggt gag gag atc ctg ttg gac acc cag 2835
Ala His Gly Glu Asp Asp Gly Glu Glu Ile Leu Leu Asp Thr Gln
935 940 945

tgc cag ggg ctg cct cgc ccg gaa ccc cag gag gcc tgc agc ctg 2880
Cys Gln Gly Leu Pro Arg Pro Glu Pro Gln Glu Ala Cys Ser Leu
950 955 960

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Glu Pro Cys Pro Pro Arg Trp Lys Val Met Ser Leu Gly Pro Cys

965 970 975

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Ser Ala Ser Cys Gly Leu Gly Thr Ala Arg Arg Ser Val Ala Cys

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Val Gln Leu Asp Gln Gly Gln Asp Val Glu Val Asp Glu Ala Ala

995 1000 1005

tgt gcg gcg ctg gtg cgg ccc gag gcc agt gtc ccc tgt ctc att 3060

Cys Ala Ala Leu Val Arg Pro Glu Ala Ser Val Pro Cys Leu Ile

1010 1015 1020

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Ala Asp Cys Thr Tyr Arg Trp His Val Gly Thr Trp Met Glu Cys

1025 1030 1035

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Ser Val Ser Cys Gly Asp Gly Ile Gln Arg Arg Arg Asp Thr Cys

1040 1045 1050

ctc gga ccc cag gcc cag gcg cct gtg cca gct gat ttc tgc cag 3195

Leu Gly Pro Gln Ala Gln Ala Pro Val Pro Ala Asp Phe Cys Gln

1055 1060 1065

cac ttg ccc aag ccg gtg act gtg cgt ggc tgc tgg gct ggg ccc 3240

His Leu Pro Lys Pro Val Thr Val Arg Gly Cys Trp Ala Gly Pro

1070 1075 1080

tgt gtg gga cag ggt gcc tgt ggc agg cag cac ctt gag cca aca 3285

Cys Val Gly Gln Gly Ala Cys Gly Arg Gln His Leu Glu Pro Thr

1085 1090 1095

gga acc att gac atg cga ggc cca ggg cag gca gac tgt gca gtg 3330

Gly Thr Ile Asp Met Arg Gly Pro Gly Gln Ala Asp Cys Ala Val

1100 1105 1110

gcc att ggg cgg ccc ctc ggg gag gtg gtg acc ctc cgc gtc ctt 3375

Ala Ile Gly Arg Pro Leu Gly Glu Val Val Thr Leu Arg Val Leu

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Glu Ser Ser Leu Asn Cys Ser Ala Gly Asp Met Leu Leu Leu Trp

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Gly Arg Leu Thr Trp Arg Lys Met Cys Arg Lys Leu Leu Asp Met

1145 1150 1155

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Thr Phe Ser Ser Lys Thr Asn Thr Leu Val Val Arg Gln Arg Cys

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1175 1180 1185

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Ala Pro Glu Thr Phe Tyr Arg Glu Cys Asp Met Gln Leu Phe Gly

1190 1195 1200

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Glu Gly Ala Asn Ala Ser Tyr Ile Leu Ile Arg Asp Thr His Ser

1250 1255 1260

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Leu Arg Thr Thr Ala Phe His Gly Gln Gln Val Leu Tyr Trp Glu

1265 1270 1275

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1280 1285 1290

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Lys Ala Gln Ala Ser Leu Arg Gly Gln Tyr Trp Thr Leu Gln Ser

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gga acc 3966

Gly Thr

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1 5 10 15

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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val

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Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
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Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
140 145 150
gag att ggg cac agc ttc ggc ctg gag cac gac ggc gcg ccc ggc 495
Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly
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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala

170 175 180

gcg ccc cgc gcc ggc ctc gcc tgg tcc ccc tgc agc cgc cgg cag 585

Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln

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Leu Leu Ser Leu Leu Arg Thr Gly Ala Leu Arg Val Gly Pro Ala

200 205 210

gcg gcc tca acc cgg gtc cgc ggg gca ccc gcc gga tgc gca gcc 675

Ala Ala Ser Thr Arg Val Arg Gly Ala Pro Ala Gly Cys Ala Ala

215 220 225

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Trp Pro Leu Leu Gln Arg Gln Arg Ala Val Pro Arg Gly Leu Arg

230 235 240

ccc caa ggc tgt cgc ctg cac ctt cgc cag gga gca cct gga tat 765

Pro Gln Gly Cys Arg Leu His Leu Arg Gln Gly Ala Pro Gly Tyr

245 250 255

gtg cca ggc cct ctc ctg cca cac aga ccc gct gga cca aag cag 810

Val Pro Gly Pro Leu Leu Pro His Arg Pro Ala Gly Pro Lys Gln

260 265 270

ctg cag ccg cct cct cgt tcc tct cct gga tgg gac aga atg tgg 855

Leu Gln Pro Pro Pro Arg Ser Ser Pro Gly Trp Asp Arg Met Trp

275 280 285

cgt gga gaa gtg gtg ctc caa ggg tcg ctg ccg ctc cct ggt gga 900

Arg Gly Glu Val Val Leu Gln Gly Ser Leu Pro Leu Pro Gly Gly

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gct gac ccc cat agc agc agt gca tgg gcg ctg gtc 936

Ala Asp Pro His Ser Ser Ser Ala Trp Ala Leu Val

305 310

<210>21

<211>270

<212>PRT

<213> Homo sapiens

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1 5 10 15

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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val
20 25 30

ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc 135
Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
35 40 45

ctg ggg gct cag ttt cgg gtg cac ctg gtg aag atg gtc att ctg 180
Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
50 55 60

aca gag cct gag ggt gct cca aat atc aca gcc aac ctc acc tcg 225
Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser
65 70 75

tcc ctg ctg agc gtc tgt ggg tgg agc cag acc atc aac cct gag 270
Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
80 85 90

gac gac acg gat cct ggc cat gct gac ctg gtc ctc tat atc act 315
Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
95 100 105

agg ttt gac ctg gag ttg cct gat ggt aac cgg cag gtg cgg ggc 360
Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
110 115 120
gtc acc cag ctg ggc ggt gcc tgc tcc cca acc tgg agc tgc ctc 405
Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
125 130 135
att acc gag gac act ggc ttc gac ctg gga gtc acc att gcc cat 450
Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
140 145 150
gag att ggg cac agc ttc ggc ctg gag cac gac ggc gcg ccc ggc 495
Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly
155 160 165
agc ggc tgc ggc ccc agc gga cac gtg atg gct tcg gac ggc gcc 540
Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala
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gcg ccc cgc gcc ggc ctc gcc tgg tcc ccc tgc agc cgc cgg cag 585
Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln
185 190 195
ctg ctg agc ctg ctc aga ccc gtc cct ccg tcg ccg ctc cct ctg 630
Leu Leu Ser Leu Leu Arg Pro Val Pro Pro Ser Pro Leu Pro Leu
200 205 210
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Leu Ala Thr His Leu Cys Ala Gly Arg Ser Leu Ser Leu Gly Pro
215 220 225
agc caa gag ccg gct cct ggt ggg ggg cgc ggg ccg aga act cct 720
Ser Gln Glu Pro Ala Pro Gly Gly Gly Arg Gly Pro Arg Thr Pro
230 235 240
gtt ccc act cac aaa agg cca cgc ttc caa acg ctt cca tcc tcg 765

Val Pro Thr His Lys Arg Pro Arg Phe Gln Thr Leu Pro Ser Ser

245 250 255

tgc cca ctc ctc cgt ccc gcc tcc tcc cgg tgt aca ccc cgg gac 810

Cys Pro Leu Leu Arg Pro Ala Ser Ser Arg Cys Thr Pro Arg Asp

260 265 270

FIG. 1

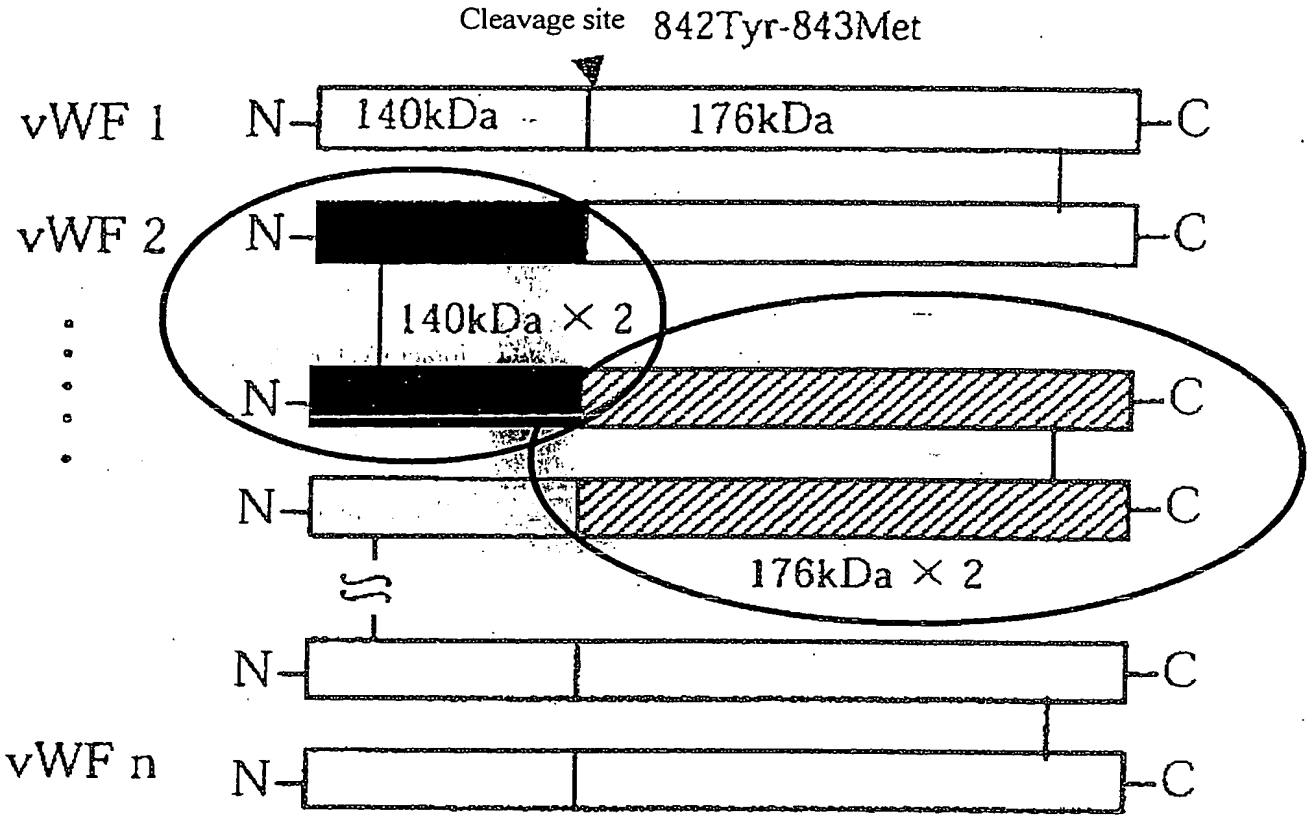
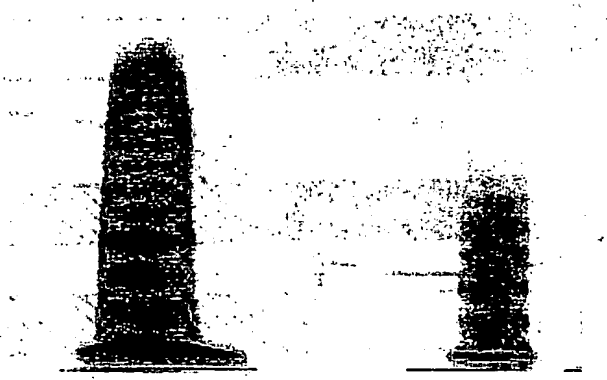

Cleavage site 842Tyr-843Met
vWF 1
N
140kDa
176kDa
C
vWF 2
N
C
140kDa × 2
N
C
N
C
176kDa × 2
N
C
vWF n
N
C

FIG. 2



Normal human plasma

Purified vWF

FIG. 3

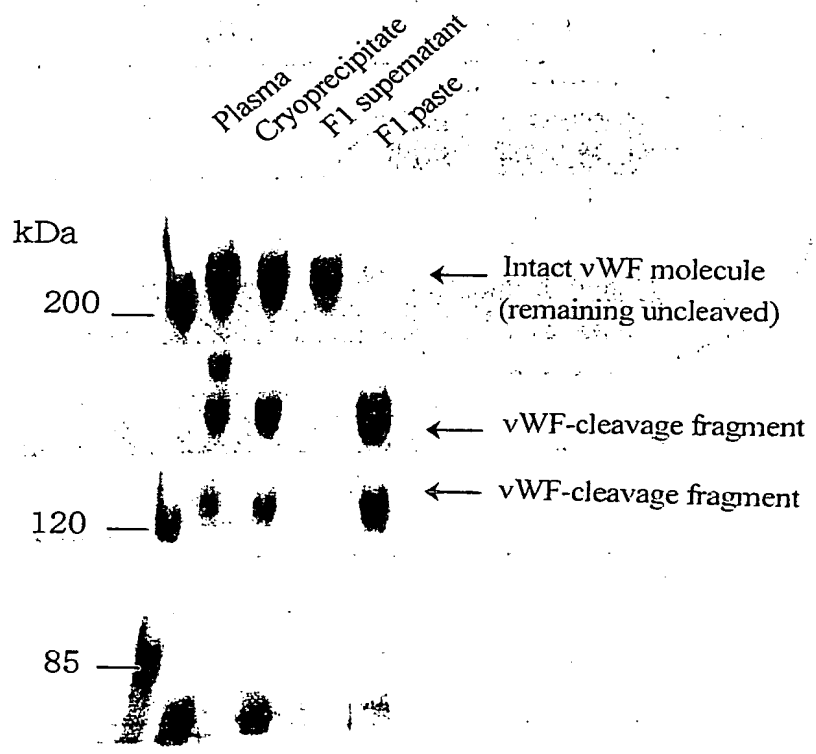

Plasma
Cryoprecipitate
F1 supernatant
F1 paste
kDa
200
120
85
Intact vWF molecule
(remaining uncleaved)
vWF-cleavage fragment
vWF-cleavage fragment

FIG. 4

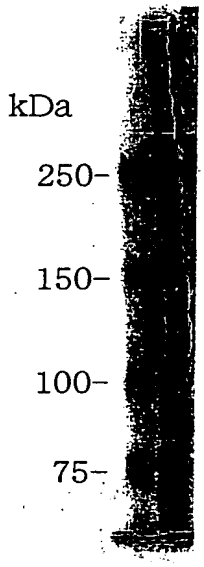


FIG. 5A

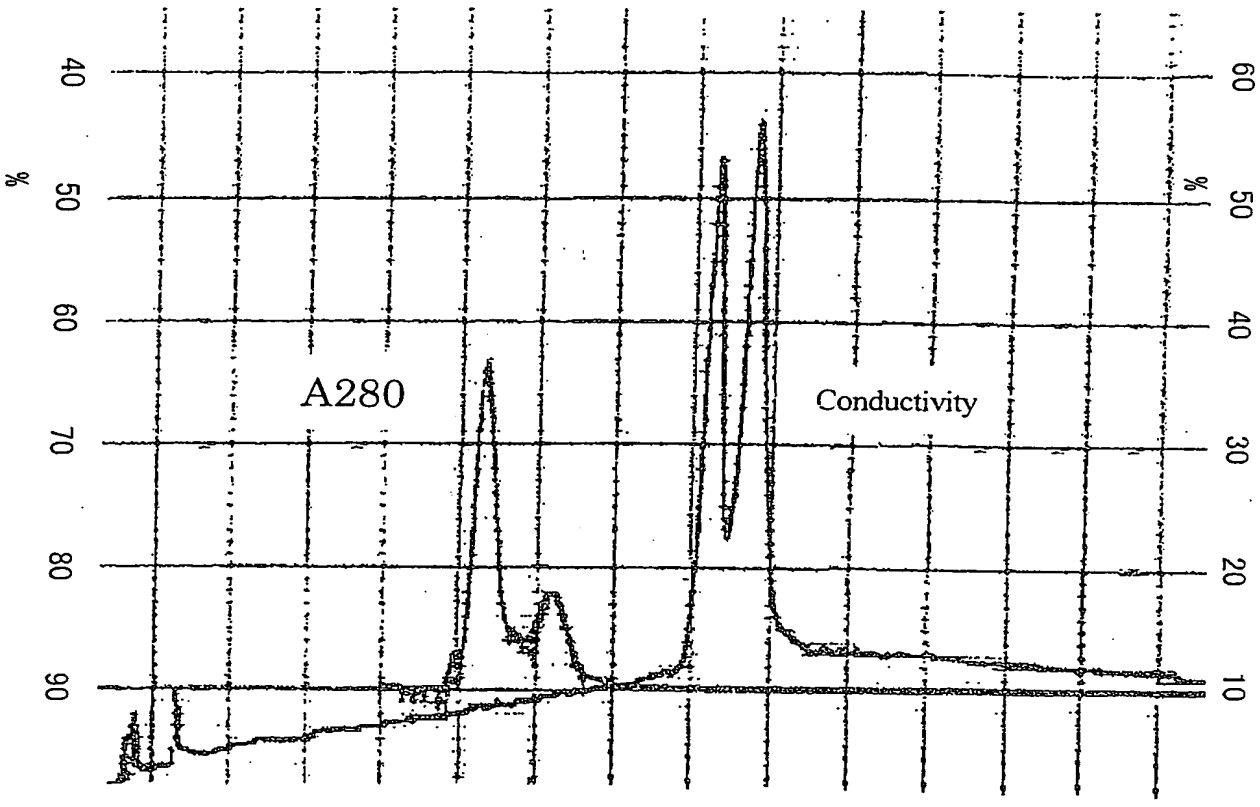
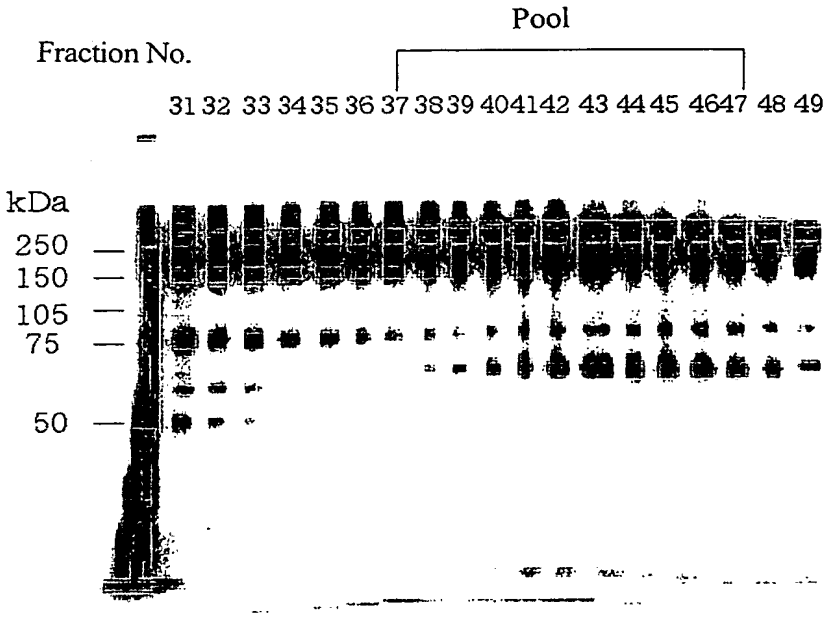


FIG. 5B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Fraction No.
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kDa
250
150
105
75
50

FIG. 5C

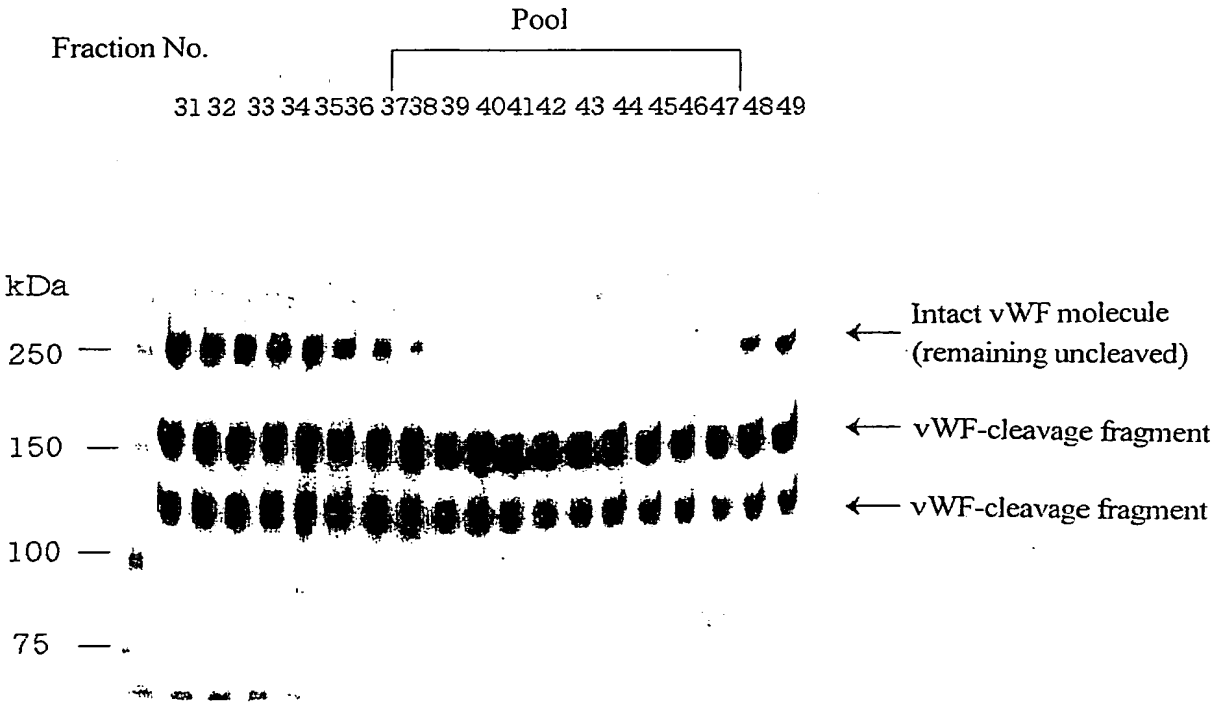

Pool
Fraction No.
31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49
kDa
250
150
100
75
Intact vWF molecule
(remaining uncleaved)
vWF-cleavage fragment
vWF-cleavage fragment

FIG. 6A

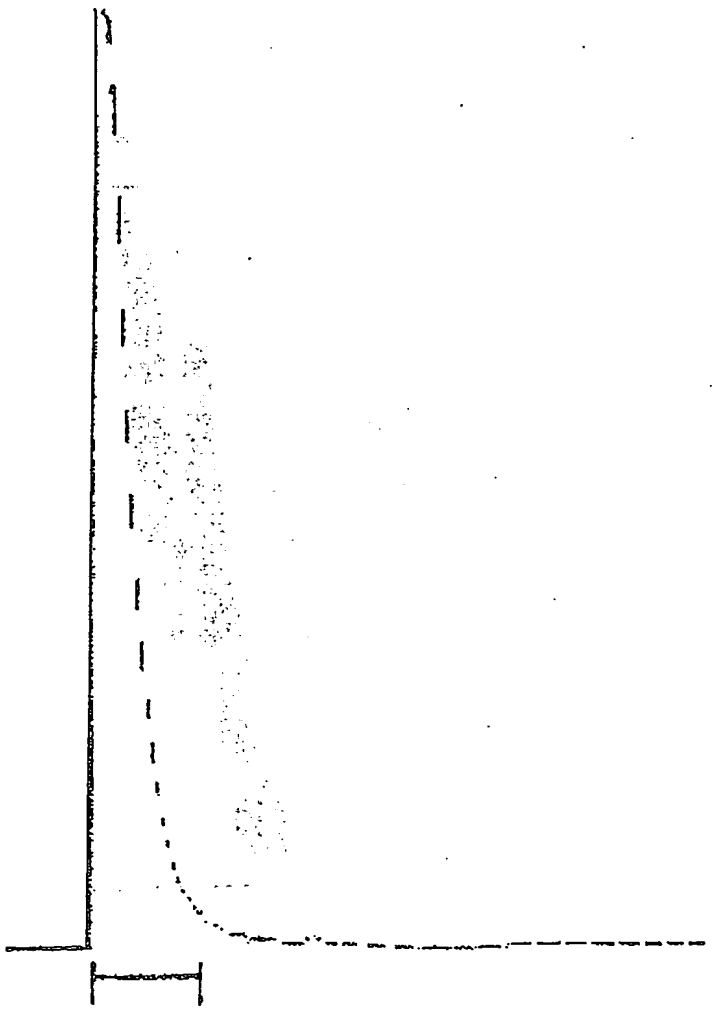


FIG. 6B

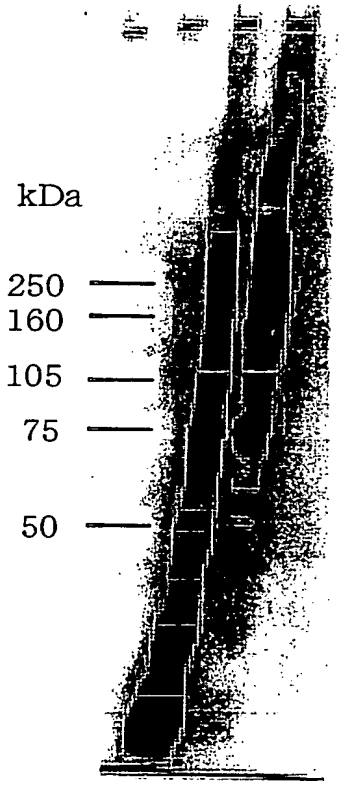


FIG. 6C

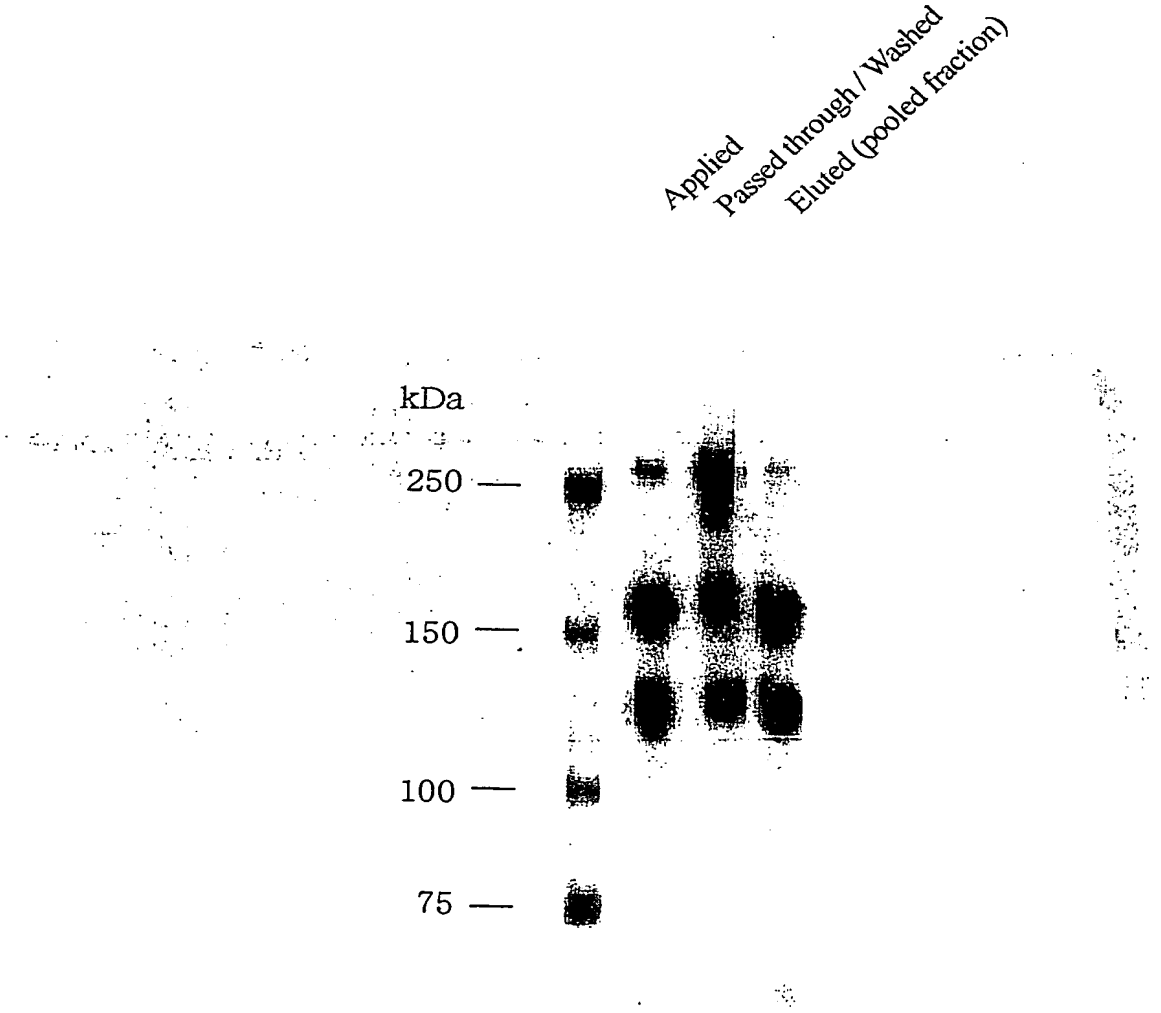

Applied
Passed through / Washed
Eluted (pooled fraction)
kDa
250
150
100
75

FIG. 7

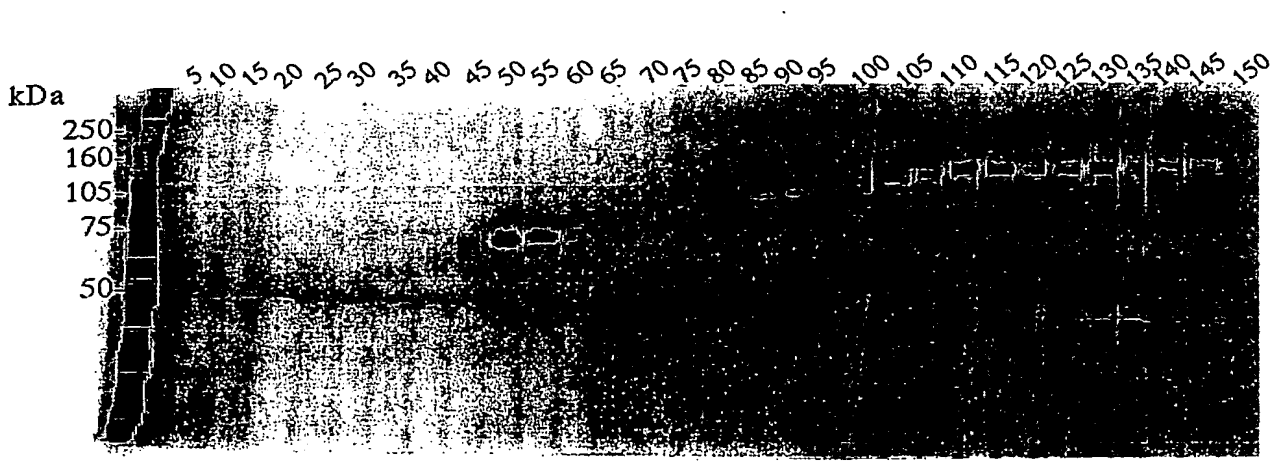

Fraction No.
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150
kDa
250
160
105
75
50

FIG. 8A

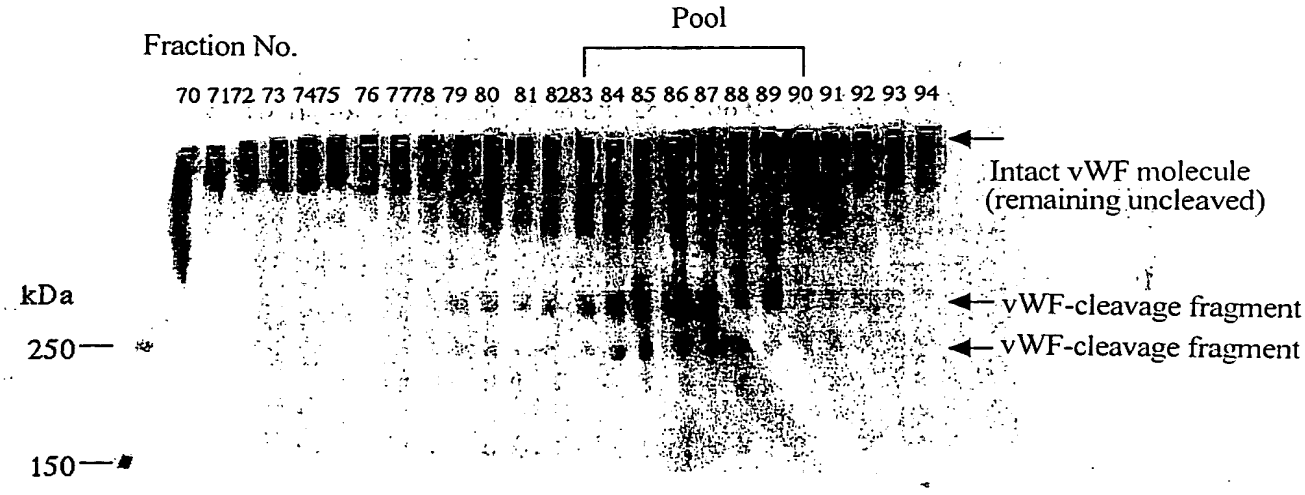

Pool
Fraction No.
70 7172 73 7475 76 7778 79 80 81 8283 84 85 86 87 88 89 90 91 92 93 94
Intact vWF molecule
(remaining uncleaved)
kDa
250
150
vWF-cleavage fragment
vWF-cleavage fragment

FIG. 8B

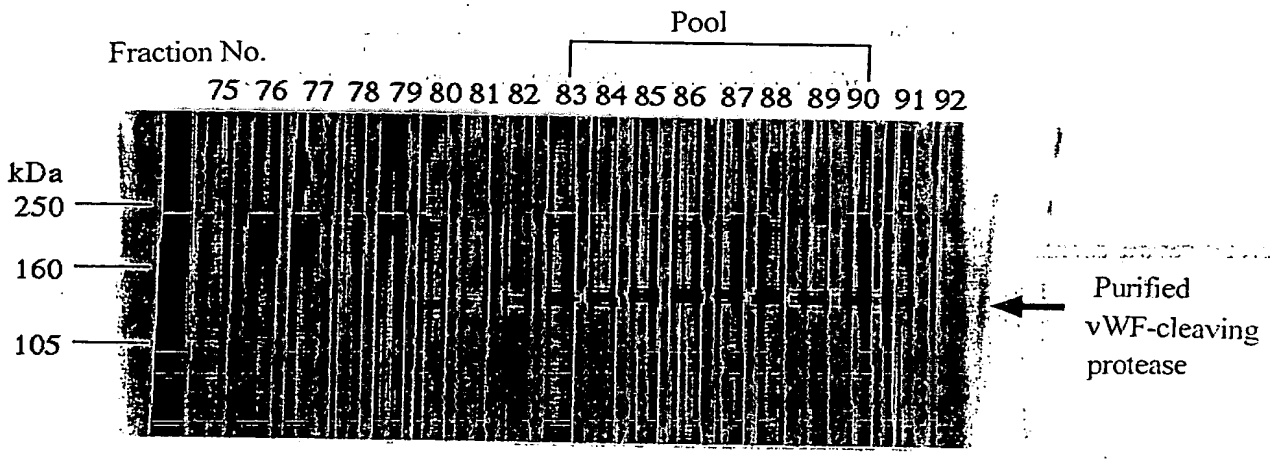
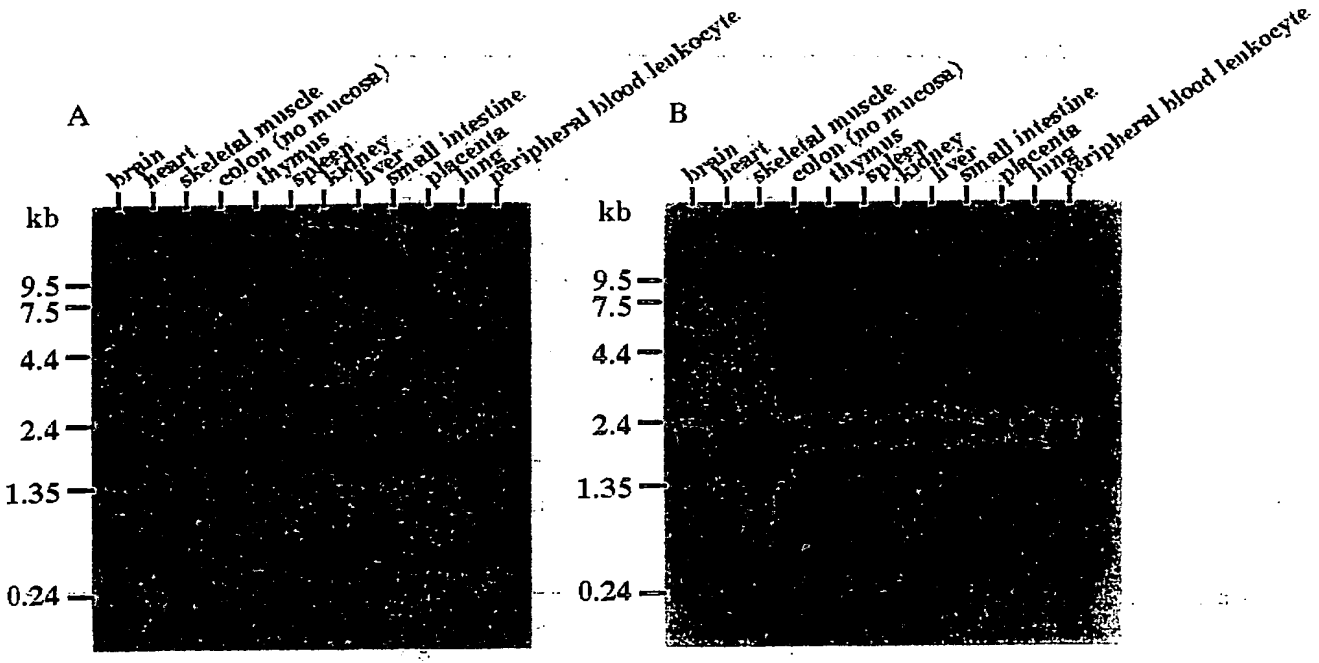

Pool
Fraction No.
75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92
kDa
250
160
105
Purified
vWF-cleaving
protease

FIG. 9

```
gct gca ggc ggc atc cta cac ctg gag ctg ctg gtg gcc gtg ggc
Ala Ala Gly Gly Ile Leu His Leu Glu Leu Leu Val Ala Val Gly
1               5                  10                  15
ccc gat gtc ttc cag gct cac cag aag gac aca gag cgc tat gtg
Pro Asp Val Phe Gln Ala His Gln Lys Asp Thr Glu Arg Tyr Val
            20                  25                  30
ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc
Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
        35                  40                  45
ctg ggg gct cag ttt cgg gtg cac ctg gtg aag atg gtc att ctg
Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
    50                  55                  60
aca gag cct gag ggt gct cca aat atc aca gca aac ctc acc tcg
Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser
65                  70                  75
tcc ctg ctg agc gtc tgt ggg tgg agc cag acc atc aac cct gag
Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
            80                  85                  90
gac gac acg gat cct ggc cat gct gac ctg gtc ctc tat atc act
Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
        95                  100                 105
agg ttt gac ctg gag ttg cct gat ggt aac cgg cag gtg cgg ggc
Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
    110                 115                 120
gtc acc cag ctg ggc ggt gcc tgc tcc cca acc tgg agc tgc ctc
Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
125                 130                 135
att acc gag gac act ggc ttc gac ctg gga gtc acc att gcc cat
Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
            140                 145                 150
gag att ggg cac agc ttc ggc ctg gag cac gac
Glu Ile Gly His Ser Phe Gly Leu Glu His Asp
        155                 160
```

FIG. 10



brain
heart
skeletal muscle
colon (no mucosa)
thymus
spleen
kidney
liver
small intestine
placenta
lung
peripheral blood leukocyte

FIG. 11

gctgcaggcg gcatcctaca cctggagctg ctggtggccg tgggccccga tgtcttccag
Primer 1
gctcaccaga aggacacaga gcgctatgtg ctcaccaacc tcaacatcgg ggcagaactg
Primer 3
cttcgggacc cgtccctggg ggctcagttt cgggtgcacc tggtgaagat ggtcattctg

acagagcctg agggtgctcc aaatatcaca gcaaacctca cctcgtccct gctgagcgtc

tgtgggtgga gccagaccat caaccctgag gacgacacgg atcctggcca tgctgacctg
Primer 4
gtcctctata tcactaggtt tgacctggag ttgcctgatg gtaaccggca ggtgcggggc

gtcacccagc tgggcggtgc ctgctcccca acctggagct gcctcattac cgaggacact

ggcttcgacc tgggagtcac cattgcccat gagattgggc acagcttcgg cctggagcac
Primer 2
gac

Primer 1
Sense : gctgcaggcg gcatcctaca cctggagctg
Antisense : cagctccagg tgtaggatgc cgcctgcagc

Primer 2
Sense : accattgccc atgagattgg g
Antisense : cccaatctca tgggcaatgg t

Primer 3
Sense : gcgctatgtg ctcaccaacc tcaacatcgg
Antisense : ccgatgttga ggttggtgag cacatagcgc

Primer 4
Sense : atcaaccctg aggacgacac
Antisense : gtgtcgtcct cagggttgat